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Effect of glucose on the expression of the angiotensinogen gene in opossum kidney cells

TIAN-TIAN WANG, XIAO-HUA WU, SHAO-LING ZHANG, AND JOHN S.D. CHAN

University of Montreal, Maisonneuve-Rosemont Hospital, Research Center, Montreal, Quebec, Canada

Effect of glucose on the expression of the angiotensinogen gene in opossum kidney cells. To investigate whether D(+)-glucose has a stimulatory effect on the expression of the angiotensinogen (Ang) gene in opossum kidney (OK) cells, we used OK cells with a fusion gene containing various lengths of the 5′-flanking regulatory sequence of the rat Ang gene fused with the human growth hormone (hGH) gene as a reporter, stably integrated into their genomes. The level of expression of the fusion gene was quantified by the amount of immunoreactive-human growth hormone (IR-hGH) secreted into the medium. The addition of D(+)-glucose stimulated the expression of pOGH (Ang N-1498/+18) in OK 27 cells in a dose-dependent manner (5 to 25 mM), whereas the addition of D-mannitol, L-glucose and 2-deoxy-D-glucose (25 mM) had no effect. The stimulatory effect of D(+)-glucose (25 mM) was blocked by the presence of staurosporine or H7 (an inhibitor of protein kinase C) or U73122 (an inhibitor of phospholipase C and A_2) but not blocked by the presence of Rp-cAMP (an inhibitor of cAMP-dependent protein kinase A). The addition of D(+)-glucose (25 mM) also stimulated the expression of pOGH (Ang N-960/+18) and pOGH (Ang N-688/+18) in OK 960 and OK 688 cells, respectively. It had no stimulatory effect, however, on the expression of pOGH (Ang N-280/+18) and pOGH (Ang N-35/+18) in OK 280 and OK 35 cells, respectively. The addition of D(+)-glucose also had no effect on the expression of pTKGH in OK 13 cells, an OK cell line, into which had been stably integrated a fusion gene, pTKGH containing the promoter/enhancer DNA sequence of the viral thymidine-kinase (TK) gene fused with a human growth hormone gene as a reporter. These studies demonstrate that the stimulatory effect of high D(+)-glucose concentration (25 mM) on the expression of the angiotensinogen-growth hormone fusion genes in OK cells is mediated via the 5′-flanking region of the angiotensinogen gene and the protein kinase C signal transduction pathway. Our data indicate that a high glucose concentration may activate the renin-angiotensin system in the renal proximal tubular cells.

Studies have shown that the mRNA components of the renin-angiotensin system (RAS), including angiotensinogen (Ang), renin, angiotensin-converting enzyme (ACE) and angiotensin-II receptor (AT_1-receptor) are expressed in murine (mouse and rat) immortalized proximal tubular cell lines [1–4]. More recently, we have reported that the Ang protein is secreted from rat immortalized proximal tubular cells as measured by a specific radioimmunoassay for rat Ang [5]. We [6] as well as Ingelfinger et al [7] have also demonstrated that the Ang mRNA is expressed in opossum kidney (OK) proximal tubular cells. These studies indicate that the intrarenal angiotensin II (Ang II) is probably derived from the Ang that is synthesized within the renal proximal tubular cells.

We have previously reported that isoproterenol and iodoclonidine stimulate the expression of the Ang gene in OK cells *in vitro* in a dose-dependent manner [8, 9]. The effect of isoproterenol is mediated via the β_1-adrenoceptor and cAMP-dependent protein kinase A (PKA) pathway [8], whereas the effect of iodoclonidine is mediated via the α_2-adrenoceptor and protein kinase C (PKC) pathway [9]. Our studies confirm the reports of Nakamura and Johns [10] that low levels of renal nerve stimulation increase the Ang mRNA levels in the rat kidney *in vivo*. Our studies and those of Nakamura and Johns [8–10] together indicate the presence of a functional relationship between the renal sympathetic nervous system and the activation of local intrarenal RAS. Thus, the local formation of renal Ang II may play an important role in the physiology of the renal proximal tubular cells (that is, sodium and fluid reabsorption [11–14]).

Hyperglycemia is an important etiologic factor in the development of diabetic nephropathy [15]. However, the molecular mechanisms for the development of nephropathy in diabetes are not completely understood. Studies have shown that the incubation of mesangial cells in a high glucose medium increases cellular proliferation, extracellular matrix protein synthesis and the expression of mRNA of extracellular matrix proteins in mesangial cells [16–19]. Experimental evidence also indicates that the effect of elevated glucose concentration is probably mediated via the activation of the protein kinase C pathway in mesangial cells [20–24]. Furthermore, tubular basement membrane thickening, accumulation of glycogen droplets in proximal tubular epithelium and tubulointerstitial fibrosis are often observed in diabetic patients [25]. These studies indicate that the elevated glucose concentration may directly or indirectly be responsible for the development of diabetic nephropathy.

We have previously demonstrated that the addition of phorbol 12-myristate 13-acetate (PMA, a protein kinase C stimulator) has a stimulatory effect on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells [9], indicating that the PKC signal transduction pathway plays a regulatory role on the expression of the Ang gene in OK cells. In the present studies, we investigated the possible effect of glucose on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells. Our results showed that the expression of pOGH (Ang N-1498/+18) fusion gene in OK 27

Key words: angiotensinogen gene, glucose, opossum kidney cells, growth hormone, renin-angiotensin system.

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cells is stimulated by high concentrations (25 mM) of D(+)-glucose, but not by D-mannitol, L-glucose or 2-deoxy-D-glucose. Furthermore, the addition of staurosporine or H-7 (an inhibitor of protein kinase C) and U73122 (an inhibitor of phospholipase C and A_2) blocked the stimulatory effect of glucose. Finally, the addition of high glucose (25 mM) had a stimulatory effect on the expression of pOGH (Ang N-960/+18) and pOGH (Ang N-688/+18) in OK 960 and OK 688 cells, respectively. The addition of glucose had no effect, however, on the expression of pOGH (Ang N-280/+18), pOGH (Ang N-35/+18) and pTKGH in OK 280, OK 35 and OK 13 cells, respectively.

METHODS

D(+)-glucose, L-glucose, D-mannitol and 2-deoxy-D-glucose were purchased from Sigma Chemicals (St. Louis, MO, USA). Staurosporine (an inhibitor of protein kinase C), H-7 (an inhibitor of protein kinase C), U73122 (an inhibitor of phospholipase C and A_2) and Rp-cAMP (an inhibitor of the cAMP-dependent protein kinase AI and II) were purchased from Research Biochemicals Inc. (RBI, Natick, MA, USA).

The plasmid, pRSV-Neo, containing the coding sequence for Neomycin (Neo) with the Rous Sarcoma Virus (RSV) enhancer/promoter sequence fused in the 5′-end of the Neomycin gene was a gift from Dr. Teresa Wang (Dept. of Pathology, Stanford University, Stanford, CA, USA). The plasmid, pTKGH, containing the thymidine kinase (TK) enhancer/promoter sequence fused to the 5′-end of the hGH gene was purchased from the Nichols Institute of Diagnostics (La Jolla, CA, USA).

The radioimmunoassay kit for hGH (RIA-hGH) was a gift from NIADDK, NIH, USA. The RIA procedure has been described previously [26]. NIAMDD-hGH-I-1 (AFP-4793 B) was used for both iodination and as a hormone standard. The limit of sensitivity of the assay was 0.1 ng/ml. The inter- and intra-assay coefficients of variation were 10% (n-10) and 12% (n-10), respectively.

$Na^{125}I$ was purchased from Dupont, New England Nuclear (NEN, Boston, MA, USA). Calcium chloride was purchased from Mallinckrodt, Inc. (Montreal, Quebec, Canada), Geneticin (G 418) was purchased from Bethesda Research Laboratories (Gibco-BRL, Burlington, Ontario, Canada). Other molecular biology grade reagents were obtained either from Sigma Chemicals, Gibco-BRL, Boehringer-Mannheim, Pharmacia Inc. (Baie d'Urfe, Quebec, Canada), or Promega-Fisher, Inc. (Montreal, Quebec, Canada).

Construction of fusion genes

The method of construction of the Ang-GH fusion genes, pOGH (Ang N-1498/+18) and pOGH (Ang N-35/+18), has been described previously [26]. To construct pOGH (Ang N-960/+18) and pOGH (Ang N-280/+18), we simply transferred the DNA fragments, Ang N-960/+18 and Ang N-280/+18, from the plasmids pOCAT (Ang N-960/+18) and pOCAT (Ang N-280/+18) [27], respectively, into the pOGH vector.

Cell culture

The opossum kidney (OK) proximal tubular cell line was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). This cell line is derived from the kidney of a female American opossum and retains several properties of proximal tubular epithelial cells in culture [28, 29] and expresses a low level of Ang mRNA [6, 7]. The culture conditions of OK cells have been described previously [8, 9, 30].

Opossum kidney cell stable transformants

Opossum kidney 27 and OK 13 cells are stable transformants with pOGH (Ang N-1498/+18) and pTKGH integrated into OK cellular genomes, respectively. The characteristics of these cells have been previously reported [8, 9]. Briefly, OK 27 and OK 13 cells that had passed through at least three repetitions of limiting dilution and continued to secrete high levels of immunoreactive-hGH (IR-hGH) after three months in the presence of G418 were considered to be stable clones.

By similar procedures, we have obtained stable transformants OK 960, OK 688, OK 280 and OK 35 with pOGH (Ang N-960/+18), pOGH (Ang N-688/+18), pOGH (Ang N-280/+18) and pOGH (Ang N-35/+18) integrated into OK cellular genomes, respectively. The expression of the Ang-GH fusion genes in these cells was time-dependent. The levels of IR-hGH in cellular extracts were consistently less than 5% of those found in the culture media, suggesting that renal Ang is not stored in the cell.

Effect of D(+)-glucose on the expression of angiotensinogen-growth hormone fusion genes in opossum kidney 27 cell stable transformants

Opossum kidney 27 cells were plated at a density of 1 to 2×10^5 cells/well in six-well plates and incubated overnight in DMEM containing 10% FBS. Cell growth was then arrested by incubation in serum-free and a low D(+)-glucose concentration (5 mM) medium for 24 hours. Subsequently, various concentrations of D(+)-glucose (final concentration 5 to 25 mM) were added to the culture medium containing 1% depleted fetal bovine serum (dFBS) and incubated for 24 hours. Since glucose changes the tonicity of the media, the media were supplemented with D-mannitol until the final concentration was reached equivalent 30 mM (that is, 5 mM D(+)-glucose plus D-mannitol until equivalent 30 mM, etc.). At the end of the incubation period, media were collected and kept at −20°C until assayed for IR-hGH.

To determine the specificity of D(+)-glucose, 5 or 25 mM of L-glucose or D-mannitol or 2-deoxy-glucose was added to the culture medium and incubated for 24 hours. Then, the media were collected and kept at −20°C until assay for IR-hGH.

To compare the inhibitory effect of staurosporine, H-7, U73122 and Rp-cAMP on the expression of pOGH (Ang N-1498/+18) in OK 27 cells, various concentrations (10^{-13} to 10^{-7} M) of the inhibitors were co-cultured with the D(+)-glucose (25 mM) for 24 hours. At the end of the incubation period, media were collected and kept at −20°C until assay for IR-hGH.

The depleted FBS was prepared by incubation with 1% activated charcoal and 1% AG 1 × 8 ion-exchange resin (Bio-Rad Laboratories, Richmond, CA, USA) for 16 hours or more at room temperature as described by Samuels, Stanley and Shapiro [31]. This procedure removed endogenous steroid and thyroid hormones from the FBS as demonstrated by Samuels et al [31].

Statistical analysis

The experiments were performed at least three to four times in triplicate. The data were analyzed with Student's *t*-test or analysis of variance (ANOVA). A probability level of $P \leq 0.05$ was regarded as significant.

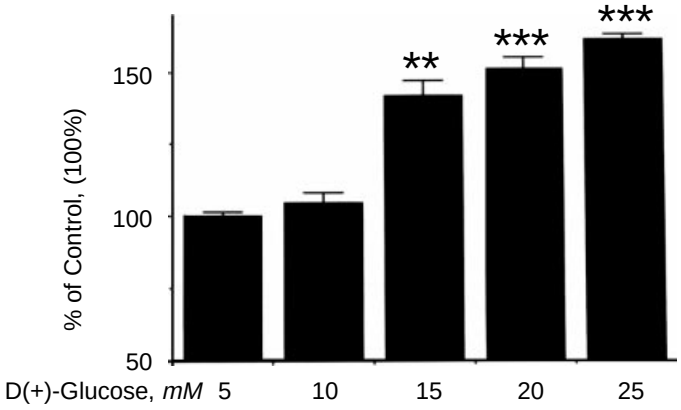

Fig. 1. Effect of D(+)-glucose on the expression of pOGH (Ang N-1498/+18) in opossum kidney (OK) 27 cells. Cells were incubated for up to 24 hours in the presence of various concentrations of D(+)-glucose. Media were collected after 24 hours of incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the medium containing low D(+)-glucose (5 mM) (that is, 1.43 ± 0.1 ng/ml) is considered as the control level. Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Experiments were repeated three times.

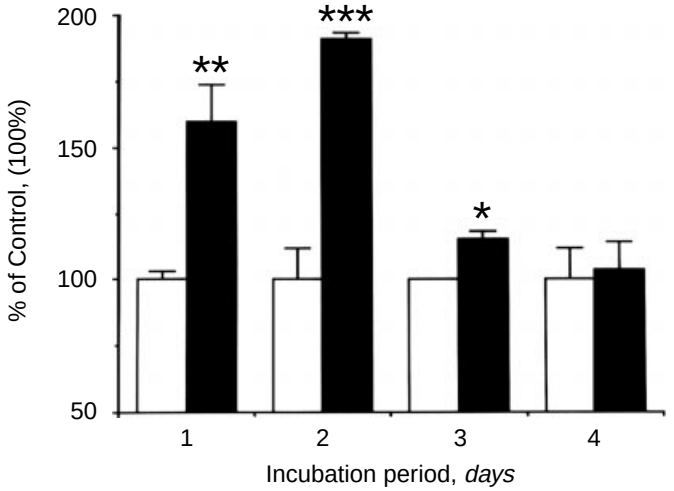

Fig. 2. Effect of incubation period on the expression of pOGH (Ang N-1498/+18) in opossum kidney (OK) 27 cells. Cells were incubated for one to four days in the presence of 5 mM or 25 mM of D(+)-glucose. Media were harvested after various periods of incubation and assayed for IR-hGH. The concentration of IR-hGH in the medium containing low D(+)-glucose (5 mM) (that is, Day 1, 1.32 ± 0.1 ng/ml; Day 2, 1.61 ± 0.15 ng/ml; Day 3, 1.86 ± 0.15 ng/ml; Day 5, 1.91 ± 0.1 ng/ml) is considered as the control level. Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from two other experiments.

RESULTS

Effect of D(+)-glucose on the expression of angiotensinogen-growth hormone fusion gene in opossum kidney cell stable transformants

Figure 1 shows the expression of the pOGH (Ang N-1498/+18) in OK 27 cells in the presence of various concentrations (5 to 25 mM) of D(+)-glucose after 24 hours of incubation. A dose-dependent relationship between D(+)-glucose concentrations and the stimulation of expression of pOGH (Ang N-1498/+18) was observed at 5 to 25 mM. The maximal stimulation of expression of the pOGH (Ang N-1498/+18) was found with 25 mM of D(+)-glucose.

Figure 2 shows the expression of the pOGH (Ang N-1498/+18) in OK 27 cells in the presence of 5 mM or 25 mM D(+)-glucose at different time periods. The maximal expression of the pOGH (Ang N-1498/+18) with 25 mM D(+)-glucose was found after two days of incubation. The stimulatory effect of 25 mM D(+)-glucose was significantly reduced following three days of incubation. There was no significant difference between the stimulatory effect observed with 25 mM D(+)-glucose at four days of incubation compared to the 5 mM D(+)-glucose. For subsequent studies, we routinely performed the experiments following 24 hours of incubation in the presence of low or high glucose levels.

Figure 3 shows that the effect of the addition of 5 mM or 25 mM D(+)-glucose on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells when OK 27 cells were pre-incubated with 25 mM D(+)-glucose (Fig. 3A) or 5 mM (D+)-glucose in the presence of 10^{-5} M phorbol 12-myristate, 13-acetate (PMA) (Fig. 3B) for 24 hours. It is apparent that the pre-incubation with 25 mM of D(+)-glucose or 10^{-5} M PMA for 24 hours abolished the stimulatory effect of 25 mM of D(+)-glucose compared to the 5 mM D(+)-glucose level.

Figure 4 compares the effect of the addition of 5 mM or 25 mM of D(+)-glucose, D-mannitol, L-glucose or 2-deoxy-D-glucose on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells

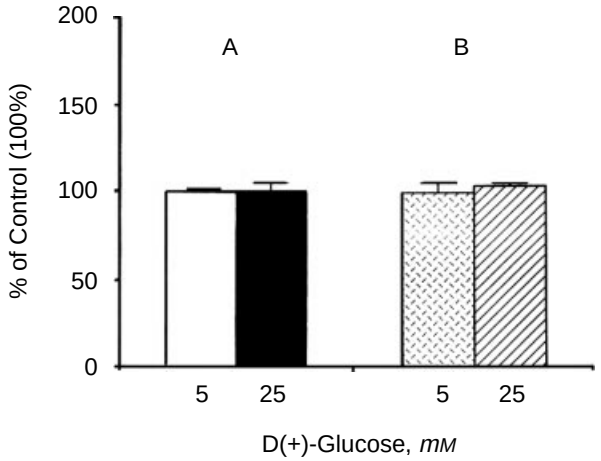

Fig. 3. Effect of the addition of D(+)-glucose on the expression of the pOGH (Ang N-1498/+18) in opossum kidney (OK) 27 cells when the cells were pre-incubated with a high level of glucose or phorbol 12-myristate 13-acetate (PMA). Cells were incubated for 24 hours with 25 mM D(+)-glucose (*A*) or 5 mM D(+)-glucose in the presence of 10^{-5} M PMA (*B*). Then, the media were replaced with the fresh media containing 5 mM or 25 mM D(+)-glucose and incubated further for 24 hours. Subsequently, the media were havested and assayed for immunoreactive-human growth hormone (IR-hGH). The concentration of IR-hGH in the medium containing 5 mM D(+)-glucose in A or B (that is, 1.80 ± 0.1 ng/ml or 1.51 ± 0.2 ng/ml) are expressed as 100% (control). Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.005$). Similar results were obtained from two other experiments.

after 24 hours of incubation with or without the supplementation of D-mannitol. In contrast to D(+)-glucose, the addition of 25 mM D-mannitol, L-glucose or 2-deoxy-D-glucose had no significant

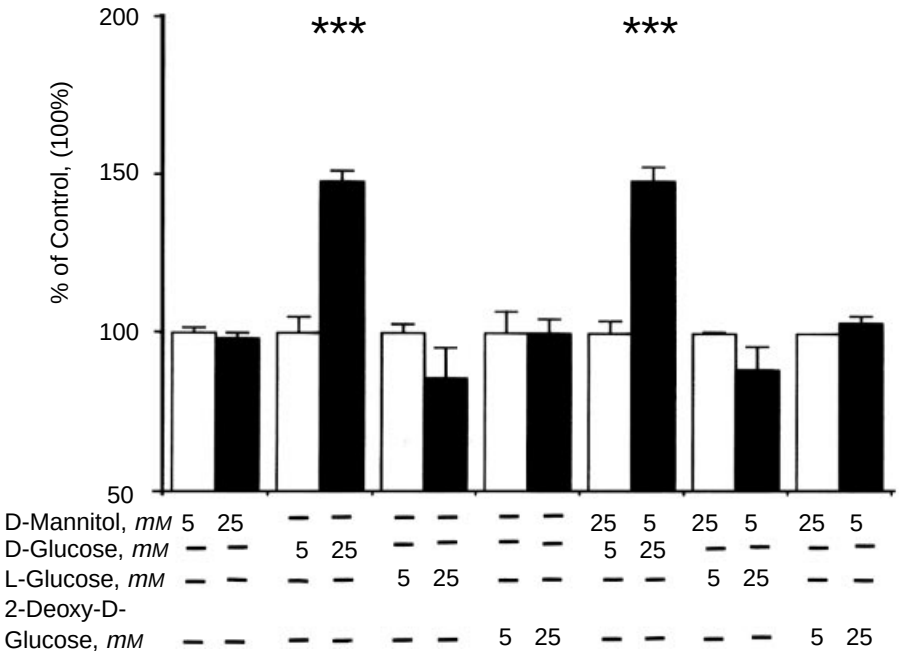

Fig. 4. Effect of D(+)-glucose, D-mannitol, L-glucose and 2-deoxy-D-glucose on the expression of pOGH (Ang N-1498/+18) in opossum kidney (OK) 27 cells. Cells were incubated for up to 24 hours in the presence of low (5 mM) or high (25 mM) of D(+)-glucose, D-mannitol, L-glucose or 2-deoxy-D-glucose with or without supplementation of D-mannitol. Media were harvested after 24 hours of incubation and assayed for immunoreactive-human growth hormone (IR-hGH). The concentration of IR-hGH in the medium containing 5 mM D(+)-glucose (4.71 ± 0.35 ng/ml), 5 mM D-mannitol (2.53 ± 0.05 ng/ml), or 5 mM L-glucose (3.32 ± 0.06 ng/ml), 5 mM 2-deoxy-D-glucose (1.42 ± 0.17 ng/ml), 5 mM D(+)-glucose plu 25 mM mannitol (2.84 ± 0.10 ng/ml), 5 mM L-glucose plus 25 mM mannitol (2.58 ± 0.02 ng/ml) or 5 mM 2-deoxy-D-glucose plus 25 mM mannitol (1.61 ± 0.01 ng/ml) are considered as the control level. Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from two other experiments.

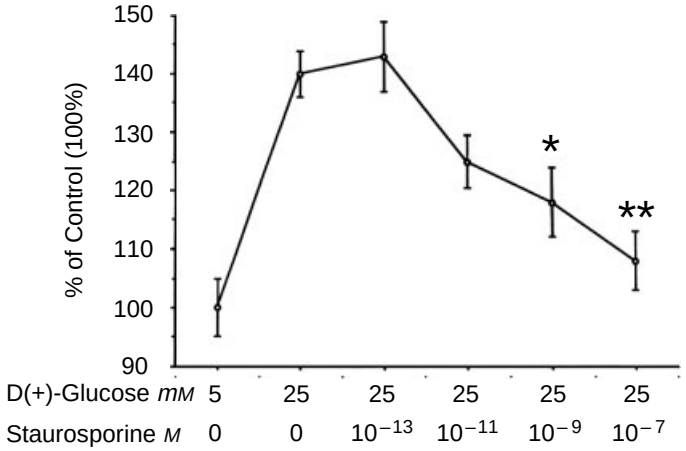

Fig. 5. Inhibitory effect of staurosporine on the expression of pOGH (Ang N-1498/+18) in OK 27 cells in the presence of 25 mM D(+)-glucose. Cells were incubated for 24 hours in the presence of 5 mM or 25 mM D(+)-glucose. Media were harvested and assayed for the level of IR-hGH. The levels of IR-hGH in the medium containing low D(+)-glucose (5 mM) (that is, 1.69 ± 0.15 ng/ml) are expressed as 100% (control). The inhibitory effect of staurosporine is compared with cells that were incubated in 25 mM D(+)-glucose (without the presence of staurosporine). Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from another experiment.

effect on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells compared to those at 5 mM.

Effect of staurosporine, H-7, U73122 or Rp-cAMP on the expression of pOGH (Ang N-1498/-18) in OK 27 cells in the presence of D(+)-glucose

Figure 5 shows that the addition of staurosporine (10^{-13} to 10^{-5} M) inhibited the stimulatory effect of D(+)-glucose (25 mM) on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells in a dose-dependent manner. The effective inhibitory dose for inhibition of the stimulated expression (25 mM D(+)-glucose) of the pOGH (Ang N-1498/+18) was at 10^{-9} M staurosporine ($P \leq 0.05$). At 10^{-7} M staurosporine, the stimulatory effect of D(+)-glucose (25 mM) on the expression of the pOGH (Ang N-1498/+18) was completely abolished.

Similarly, the addition of H-7 or U73122 (10^{-7} M) completely inhibited the stimulatory effect of D(+)-glucose on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells (Fig. 6). On the other hand, the addition of Rp-cAMP (10^{-7} M) had no inhibitory effect on the expression of pOGH (Ang N-1498/+18) stimulated by D(+)-glucose (25 mM; Fig. 6).

Effect of D(+)-glucose on the expression of the angiotensinogen-growth hormone fusion genes and pTKGH in opossum kidney cells

Figure 7 shows that the addition of 25 mM D(+)-glucose stimulated the expression of pOGH (Ang N-1498/+18), pOGH (Ang N-960/+18) and pOGH (Ang N-688/+18) in OK 27, OK 960 and OK 688 cells compared to the 5 mM D(+)-glucose, respectively. The addition of 25 mM D(+)-glucose had no stimulatory effect on the expression of pOGH (Ang N-280/+18) and pOGH (Ang N-35/-18) in OK 280 and OK 35 cells compared to the 5 mM D(+)-glucose, respectively.

Figure 8 shows that the addition of D(+)-glucose (5 to 25 mM) had no stimulatory effect on the expression of the pTKGH in OK 13 cells.

DISCUSSION

Studies *in vitro* on cultured murine proximal tubular cells in a high glucose-containing medium (that is, ≥ 25 mM) showed that the high glucose levels stimulated the hypertrophy of the proximal tubular cells [32]. It appears that the hypertrophic effect of high glucose is mediated via the autocrine induction of transforming growth factor (TGF-β) [33]. These observations were confirmed

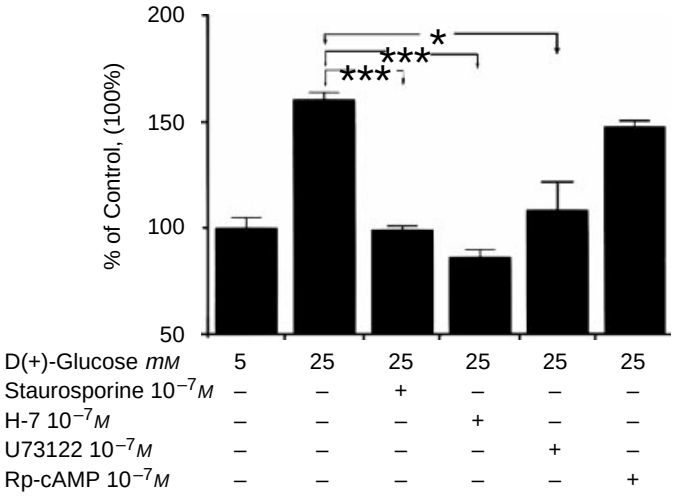

Fig. 6. Inhibitory effect of staurosporine, H-7, U73122 or Rp-cAMP on the expression of pOGH (Ang N-1498/+18) in OK 27 cells stimulated by 25 mM D(+)-glucose. Cells were incubated for 24 hours in the presence of 5 mM or 25 mM D(+)-glucose plus 10^{-7} M of staurosporine or H-7 or U73122 or Rp-cAMP. Media were harvested and assayed for the level of immunoreactive-human growth hormone (IR-hGH). Levels of IR-hGH in the medium containing the low D(+)-glucose (5 mM) (that is, 3.72 ± 0.18 ng/ml) in the absence of staurosporine, H-7, U73122 or Rp-cAMP are expressed as 100% (control). The inhibitory effect of staurosporine, H-7, U73122 or Rp-cAMP is compared with cells that were stimulated by 25 mM D(+)-glucose. Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from two other experiments.

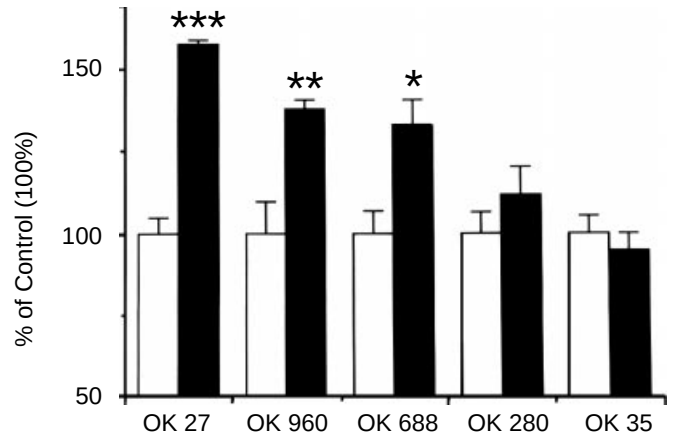

Fig. 7. Effect of D(+)-glucose on the expression of angiotensinogen-growth hormone (Ang-GH) fusion genes in opossum kidney (OK) cells. Cells were incubated for up to 24 hours in the presence of 5 mM or 25 mM D(+)-glucose. Media were harvested after 24 hours of incubation and assayed for IR-hGH. The concentration of IR-hGH in the medium containing low glucose (5 mM) (that is, OK 27 cells, 1.42 ± 0.08 ng/ml; OK 960, 1.69 ± 0.1 ng/ml; OK 688, 1.43 ± 0.1 ng/ml; OK 280, 1.28 ± 0.1 ng/ml, OK 35, 1.60 ± 0.12 ng/ml) is considered as the control level. Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from two other experiments.

by *in vivo* studies, where it was found that the renal hypertrophy is also associated with the increased renal expression of the TGF-β in spontaneously diabetic Bio-Breeding (BB) rats and non-obese diabetic rats [34].

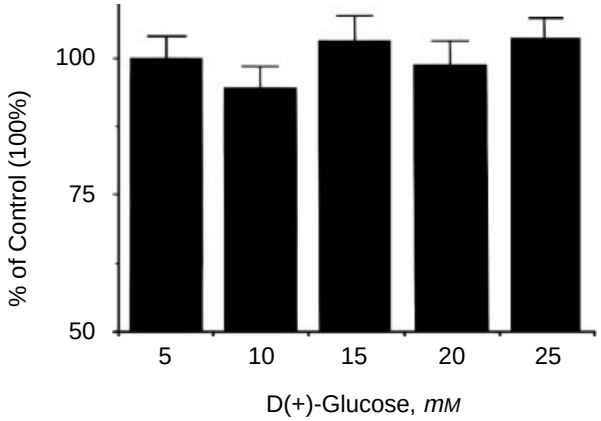

Fig. 8. Effect of D(+)-glucose on the expression of pTKGH in opossum kidney (OK) 13 cells. Cells were incubated for up to 24 hours in the presence of various concentrations of D(+)-glucose. Media were harvested after 24 hours of incubation and assayed for immunoreactive-human growth hormone (IR-hGH). The concentration of IR-hGH in the medium containing low glucose (5 mM) (that is, 1.19 ± 0.08 ng/ml) is considered as the control level. Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from two other experiments.

Studies *in vitro* also showed that the addition of Ang II stimulates the hypertrophy of mouse proximal tubular cells (MCT) and LLC-PK_1 (porcine proximal tubular cells) [2, 35–37]. The hypertrophic effect of Ang II also depends on the autocrine induction of TGF-β gene expression [38, 39]. However, studies by Wolf et al [40] demonstrated that the Ang II-mediated hypertrophy of proximal tubular cells occurs only in the presence of high concentrations of glucose. Thus, it appears that Ang II has an additive effect on the hypertrophy of the murine proximal tubular cells only in the presence of high glucose levels in the medium.

Conflicting results have been reported concerning the expression of the renin and Ang genes in the kidney in experimental diabetes mellitus. For example, studies of Kaylinyak et al [41] showed that there is no significant difference in renal renin and Ang mRNA levels in rats two weeks after the induction of diabetes compared with controls. Studies of Correa-Rotler, Hostetter and Rosenberg [42], however, reported that the levels of renal and liver Ang mRNA are lower in the diabetic group. In contrast, the studies of Everett et al [43] showed that there is an increase in immunoreactive Ang in the renal proximal tubules while there is no significant increase in renal proximal ANG mRNA in rats 4 to 8 months after induction of diabetes. The studies of Anderson, Jung and Ingelfinger [44] demonstrated that a small but significant increase is observed in renal renin and Ang mRNA expression in rats six to eight weeks after induction of diabetes with the administration of streptozotocin. Thus, until now it has been uncertain whether high glucose levels may modulate the expression of the renal Ang gene during the course of diabetes mellitus.

Our present studies showed that the addition of D(+)-glucose stimulated the expression of the fusion gene, pOGH (Ang N-1498/+18) in OK 27 cells in a dose-dependent manner (Fig. 1). Moreover, the stimulatory effect of a high D(+)-glucose level is time-dependent (Fig. 2). The highest stimulatory effect of D(+)-glucose was observed during two days of incubation (Fig. 2). The stimulatory effect was diminished with greater than two days of

incubation. We had measured the levels of glucose in the medium at various times of incubation (unpublished results). Our results showed that the levels of glucose were 0.1 mM and 15.7 mM after four days of incubation. These studies indicate that the lack of stimulatory effect by the high level of glucose following four days of incubation was not due to the depletion of glucose in the medium. We have also performed experiments by changing the old media after 48 hours of incubation with fresh media (unpublished results). The addition of fresh media did not displayed any stimulatory effect of 25 mM D(+)-glucose on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells compared to the addition of 5 mM D(+)-glucose. At present, we do not understand why longer incubation periods (that is, > two days) diminished or abolished the stimulatory effect of high D(+)-glucose on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells. One possible explanation may be that the prolonged exposure of OK cells to high D(+)-glucose may desensitize the protein kinase C signal transduction pathway. Indeed, our results (Fig. 3) showed that the 24 hours pre-incubation of OK 27 with 25 mM D(+)-glucose (Fig. 3A) or 5 mM D(+)-glucose in the presence of 10^{-5} M phorbol 12-myristate 13-acetate (PMA) (Fig. 3B) abolished the stimulatory effect of high D(+)-glucose (25 mM) on the expression of the fusion gene in OK 27 cells. Nevertheless, more experiments are warranted to clarify these observations. We did not observe any significant stimulation of the pOGH (Ang N-1498/+18) by L-glucose, D-mannitol or 2-deoxy-D-glucose (Fig. 4). These studies indicate that the effect of high D(+)-glucose levels on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells is probably mediated via the metabolized products of D(+)-glucose to stimulate the PKC pathway.

Our present studies showed that the addition of staurosporine (an inhibitor of protein kinase C) blocked the stimulatory effect of D(+)-glucose on the expression of pOGH (Ang N-1498/+18) in OK 27 cells in a dose-dependent manner (Fig. 5). Furthermore, the addition of H-7 (an inhibitor of protein kinase C) or U73122 (an inhibitor of phospholipase C and A_2) 10^{-7} M also completely blocked the stimulatory effect of high D(+)-glucose levels on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells (Fig. 6). These data support the hypothesis that the effect of high D(+)-glucose levels on the expression of the Ang gene is mediated via the protein kinase C pathway and not via the protein kinase A pathway, since Rp-cAMP (an inhibitor of cAMP-dependent protein kinase A) did not inhibit the effect of high D(+)-glucose levels on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells (Fig. 6). Indeed, the involvement of PKC on the expression of the Ang gene in OK cells are confirmed by our previous studies where it was reported that the addition of PMA stimulates the expression of the pOGH (Ang N-1498/+18) in OK 27 cells [9]. This stimulatory effect of PMA is blocked in the presence of staurosporine [9].

Our data show that the addition of high glucose (25 mM) stimulated the expression of pOGH (Ang N-1498/+18), pOGH (Ang N-960/+18) and pOGH (Ang N-688/+18) in OK 27, OK 960 and OK 688 cells, respectively (Fig. 7). The addition of high levels of glucose, however, had no effect on the expression of pOGH (Ang N-280/+18) and pOGH (Ang N-35/+18) in OK 280 and OK 35 cells, respectively. These studies indicate that the glucose-responsive element is probably localized within nucleotides N-1498 to N-280 in the 5′-flanking region of the rat Ang gene. At present, we have not identified the precise DNA sequence of the glucose-responsive element in the 5′-flanking region of the rat Ang gene. Studies are underway in our laboratory to identify the putative glucose-responsive element in the rat angiotensinogen gene.

Opossum kidney 13 is a cell line into which has been stably integrated a fusion gene: pTKGH containing the promoter/enhancer DNA sequence of the viral thymidine kinase gene fused with the human growth hormone gene as a reporter. Therefore, we used OK 13 cells as control cells to examine the effect of D(+)-glucose. We did not observe any significant stimulation of expression of the pTKGH by D(+)-glucose at various concentrations (5 to 25 mM) in OK 13 cells (Fig. 8). These data demonstrate that the promoter/enhancer DNA sequence of the TK gene is not responsive to the addition of D(+)-glucose. On the other hand, our studies demonstrated that the effect of D(+)-glucose on the expression of pOGH (Ang N-1498/+18) in OK 27 cells is gene-specific and is mediated via the 5′-flanking regulatory sequences of the rat Ang gene and not mediated via the DNA sequence of the hGH reporter gene.

At present, we do not understand the exact molecular mechanism(s) of D(+)-glucose and protein kinase C on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells. One possibility may be that the D(+)-glucose may stimulate the *de novo* synthesis of diacylglycerol (DAG) from the metabolized glucose via the polyol pathway which then increases the protein kinase C activity [45]. Surprisingly, our studies also showed that the addition of U73122 completely blocked the stimulatory effect of D(+)-glucose (Fig. 6). Since U73122 is an inhibitor of phospholipase C and A_2, these studies suggest that the glucose may indirectly increase the phospholipase C activity in OK cells by some undefined mechanism(s). Indeed, studies are underway in our laboratory to explore this possibility.

Once the PKC is activated, it is possible that the protein kinase C may phosphorylate the cAMP-responsive element binding protein (CREB) or CREB-like nuclear protein(s), since CREB contains the site of phosphorylation by protein kinase C [46] and recent studies by Kreisberg et al [47] have shown that PMA and high glucose levels stimulate the phosphorylation of CREB. The phosphorylated CREB then binds to the putative cAMP-responsive element (CRE) of the rat Ang gene (TGACGTAC, nucleotides N-795 to N-788) [26] and subsequently enhances the expression of the Ang gene. This possibility is supported by our recent studies [48] whereby the cloned CREB is able to stimulate directly the expression of the pOGH (Ang N-1498/+18) in OK 27 cells. Nevertheless, more studies are warranted to elucidate the molecular mechanism(s) of D(+)-glucose and PKC activation and the expression of the Ang gene in OK cells.

In summary, our studies show that the high D(+)-glucose levels directly stimulate the expression of the Ang-GH fusion genes in OK cells. The stimulatory effect of high D(+)-glucose concentrations was blocked by the presence of staurosporine, H-7 and U73122. Our studies suggest that the expression of the renal Ang gene may be stimulated during hyperglycemia *in vivo*. The local formation of renal Ang II might then modulate the physiology of the renal proximal tubular cells (that is, sodium and fluid reabsorption, as well as the induction of the hypertrophy of the proximal tubular cells). Thus, local renal RAS might play a significant role in the development of diabetic nephropathy.

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Reprint requests to Dr. John S.D. Chan, University of Montreal, Maisonneuve-Rosemont Hospital, Research Center, 5415 Boulevard de l'Assomption, Montreal, Quebec, Canada H1T 2M4.

APPENDIX

Abbreviations used in this article are: ACE, angiotensin converting enzyme; Ang, angiotensinogen; Ang II, angiotensin II; ANOVA, analysis of variance; MCT, mouse proximal tubular cells; AT_1-receptor, angiotensin II receptor; CRE, cAMP-responsive element; CREB, cAMP-responsive element binding protein; DAG, diacylglycerol; dFBS, depleted fetal bovine serum; hGH, human growth hormone; IR-hGH, immunoreactive-human growth hormone; LLC-PK_1, porcine proximal tubular cells; Neo, Neomycin; OK, opossum kidney; PKA, protein kinase A; pOGH (Ang N-1498/+18) and pOGH (Ang N-35/+18), angiotensinogen-growth hormone fusion genes; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RAS, renin-angiotensin system; RIA-hGH, radioimmunoassay for human growth hormone; Rp-cAMP, an inhibitor of cAMP-dependent protein kinase A; RSV, Rous Sarcoma Virus; TGF-β, transforming growth factor; TK, thymidine kinase.

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